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I further certify that the above application is now proceeding in the name of LUSTRE INVESTMENTS PTE. LTD. pursuant to the provisions of Section 113 of the Patents Act 1990.

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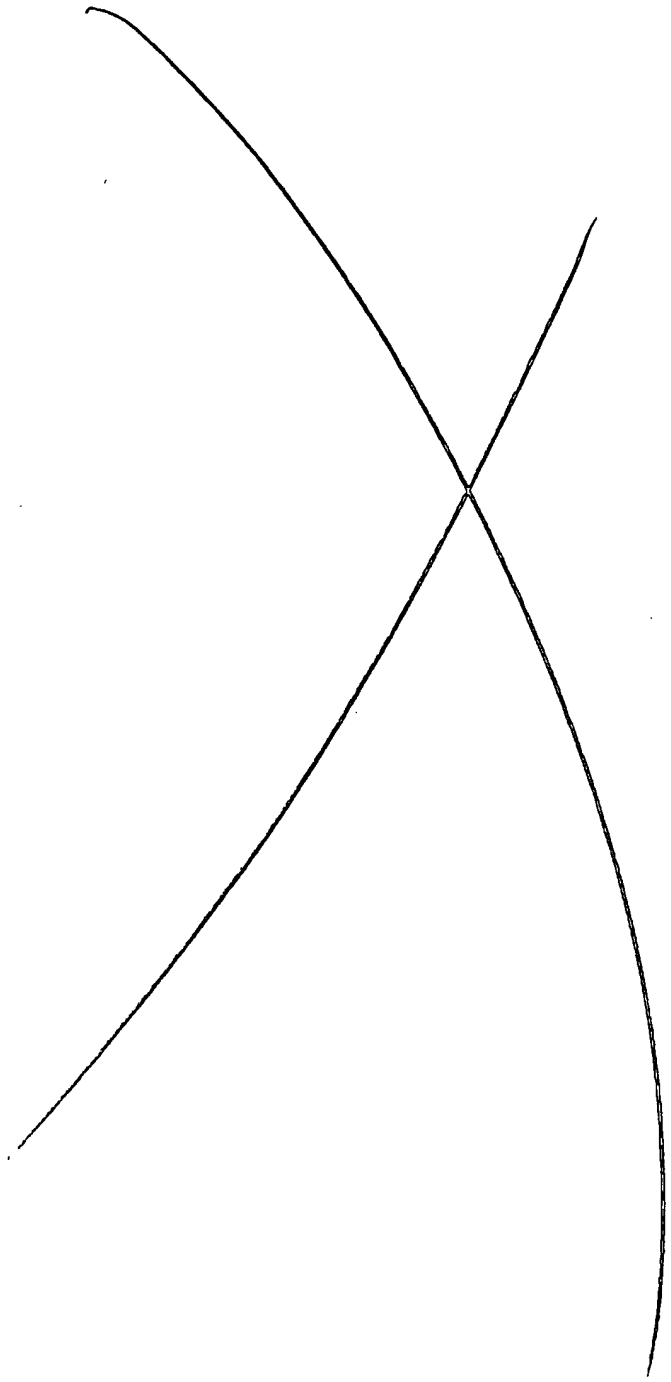

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National University of Singapore

**AUSTRALIA**

**Patents Act 1990**

**PROVISIONAL SPECIFICATION**

for the invention entitled:

"Therapeutic agents"

The invention is described in the following statement:

# THERAPEUTIC AGENTS

The present invention relates generally to therapeutic agents useful in the treatment of cancer and related disorders. The present invention further contemplates a method for ameliorating the effects and/or symptoms of cancer or related disorders in a subject by the administration of an effective amount of one or more cytokines or related molecules or genetic sequences encoding same combined with a liposomal agent.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Intravesical Bacillus Calmette-Guerin (BCG) for superficial bladder cancer is the most successful example of cancer immunotherapy today, with routine response rates of 60 -70 %[1-2]. Nevertheless two important problems exist : a significant number of patients do not respond to BCG therapy and toxicity is common[3]. Exploration of the mechanism of BCG activation of the immune response has resulted in the identification of cytokines, co-stimulatory molecules and adhesion molecules which play important roles in facilitating the cytotoxic response against tumors[4-9].

A number of studies have shown that the introduction of therapeutic genes into tumor cells can provoke an antitumor response which can lead to suppression of tumor growth[10-12]. Efficient intratumoral foreign gene delivery/transfer would therefore be a significant tool for stimulating host immune response by potentially altering tumor immunogenicity. *In vitro* transduced cytokine and viral genes expressed by tumors have resulted in the elimination of transfected

tumors and enhanced T-cell -mediated immunity to nontransduced tumors[13]. *In vivo* gene delivery would obviate the need for *in vitro* manipulations of tumor cells and enhance the clinical applicability of this therapeutic approach. Viral expression vectors have been used to introduce specific genes locally into the tumor site and thus modify the tumor into a more immunogenic form[14-15]. However, viral vectors have a number of limitations in a clinical setting such as immunogenicity and safety.

An alternative method of gene therapy *in vivo*, involves liposome-mediated delivery of DNA into cells. Brigham et al first reported delivery of cationic liposome-mediated DNA to tissues[16]. Since then cationic lipids have been shown to be efficient carriers for localized and systemic delivery of DNA to tissues *in vivo* [17-23]. Current efforts to improve the transfection efficiency of cationic liposomes are focused on the synthesis of new cationic lipids [24-26] and on the search for better lipidic formulations[27-29]. These approaches are largely based upon the study of structure-function relationships of cationic lipids and upon a better understanding about how liposomes/DNA complexes transfect cells *in vitro*.

One of the major obstacles with the use of cationic lipids for the transfection of transitional cell epithelium is the presence of a glycosaminoglycan layer which may act as a significant barrier to the uptake[30] of DNA complexes, an important determining factor in liposomal gene delivery.

In work leading up to the present invention, the inventors used water soluble cholesterol as an additive to improve the transfection of urothelial cells. The cholesterol was solubilised by packaging in methylated-β-cyclodextrin. Cyclodextrins

are oligosaccharide cyclic shaped torus- molecules which when carrying cholesterol have been shown to alter membrane fluidity and permeability. β-Cyclodextrins increase dermal and transdermal drug delivery without effecting the barrier function of skin. Cyclodextrins are used in the drug, food and cosmetic industries as additives. The inventors have now surprisingly found that the transfection efficiency is greatly enhanced by combining cationic lipid with methylated-β-cyclodextrin.

Accordingly, one aspect of the present invention provides a therapeutic agent useful in the treatment of cancer or a related disorder said agent comprising one or more cytokines or related molecules or genetic sequences encoding same and a cyclodextrin carrying a cationic lipid or a functional equivalent thereof.

Another aspect of the present invention is directed to a therapeutic agent useful in the treatment of cancer or a related disorder said agent comprising one or more cytokines or related molecules or genetic sequences encoding same and a cyclodextrin carrying cholesterol or a functional equivalent thereof.

Yet another aspect of the present invention contemplates a method of treating cancer said method comprising administering to a subject an effective amount of at least one cytokine or a related molecule or a genetic sequence encoding same together with a cyclodextrin and a lipid, such as cholesterol for a time and under conditions sufficient to inhibit growth of tumour cells or ameliorate the symptoms of the presence of the tumour.

Still another aspect of the present invention provides for the use of one or more cytokines or genetic sequences encoding same and cyclodextrin in the manufacture of a medicament for the treatment of cancer or related disorder.

The present invention is directed to the treatment of any type of cancer or condition involving uncontrolled or limited control cell proliferation. Cancers particularly

contemplated by the present invention include bladder cancer, urethral cancer, colorectal cancer, stomach cancer and pancreatic cancer.

The term "treatment of cancer" includes ameliorating the effects of cancer.

The preferred cytokines are interleukins, colony stimulating factors and interferons.

Particularly preferred interleukins are IL-1, IL-2, IL-3, IL-6, IL-9, IL-11 and IL-13. Even more particularly, the interleukin is IL-2.

Preferably colony stimulating factors are granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF). Even more particularly, the CSF is GM-CSF.

Preferred interferons are IFNα, β and γ. Even more particularly, the IFN is IFNγ.

A particularly preferred combination is selected from two or more of IL-2, GM-CSF and IFN-γ.

Most preferably, the present invention uses a dotap+methylated-β-cyclodextrin solubilised cholesterol (DMBC) to transferred cytokine genes (such as those encoding an interleukin, a CSF and/or an IFN) into tumour cells. DMBC is regarded herein as a liposomal agent.

The present invention is further described with respect to the following non-limiting Figure and/or Examples.

In the Figures:

Figure 1a: Cells transfected with DOTAP+ methylated-β-cyclodextrin containing cholesterol (DMBC) or Superfect + methylated-β-cyclodextrin containing cholesterol (SMBC) showed higher B-gal activity compared to DOTAP or Superfect alone.

Figure 1b: Percentage of cells transfected with Dotap or DMBC. B-galactosidase efficiency was measured using X-gal as described.

Figure 2a: The effect of time on protein expression following a 2 h transfection of MB49 cells with DMBC. B-gal activity was determined by ONPG assay.

Figure 2b: Duration of B-gal expression in MB49 cells. Cells were transfected as described and assayed for B-gal activity at 4, 6, 8 and 12 days later. B-gal expression was much lower after 12 days.

Figure 3: Quantification of intracellular DNA PCR. DNA extracted from MB49 cells transfected with either agents (DOTAP or DMBC). Cells seem to have internalised simillar amounts of plasmid DNA with both agents.

Figure 4: LacZ cDNA fragment (1036bp) evaluated by DNA PCR was found in both the nucleus and cytoplasm fractions of cells transfected with DMBC but only in the cytoplasm fraction of cells transfected with DOTAP.

Figure 5a and b: Toxicity of the DNA: DMBC complexes on MB49 cells *in vitro* at 2h and 24h exposure. Comparisons were made with in each case to non-transfected and relevant controls. Relative toxicity was then determined 48h later by comparing the proliferation level of each population by a [$^{14}$C]-thymidine incorporation assay.

Figure 6a and b: X-gal staining of bladder sections without and with intravesical transfection of pCMVlacZ : DOTAP+ methylated-β-cyclodextrin containing cholesterol for 2 h. Bladders were harvested 2 days later. The control bladder showed no B-galactosidase activity. In the transfected bladder epithelial cells facing the lumen were successfully transfected.(Magnification X40).

Figure 7. Absence of B-galactosidase expression in all organs except bladder confirmed by DNAPCR.

Figure 8. Bladder implanted with MB49 tumor cells were transfected with pCMVlacZ : DMBC for 2 h. Blue staining was observed in the superficial luminal cells layers of the hyperplasia (Magnification X100).

Figure 9a : The effect of shorter exposure times of DNA: DMBC complex on transfection efficiency was studied *in vitro* and *in vivo*. Cells were transfected with complex for the appropriate time periods as described.

Figure 9b: DNA: DMBC cholesterol complex transfection exposure times *in vivo*. Bladders were transfected with the optimal complex for 15, 30, 60 and 120 min) to study the effect of shorter exposure times of complex on transfection efficiency (Magnification X40).

Figure 10a and b: Immunohistochemical analyses of bladder sections following DNA: DMBC complex transfection. B-gal expression was observed as long as 30 days although expression is much lower (Magnification (X100).

Figure 11: Flow cytometric analysis of surface protein expression after cytokine gene transfection of MB-49 cells.

Figure 12: The effect of the liposomal preparation on tumour growth.

Figure 13: Effect of cytokine gene transfection given twice weekly on murine bladder tumour growth. Cytokine genes used are H-IL-2, G-IFN-γ, C-IL-2+GMCSF, D-GMCSF and F-untreated. All the genes used caused a decrease in tumour growth. Compared to the once weekly treatment given previously there was a pronounced difference between the untreated mice and those given cytokine genes as early as the 13th day after implantation of tumours. Previously a difference was only observed at 21 days post implantation.

Figure 14: Tumour burden at 37 days post implantation after 6 injections of cytokine genes compared no treatment. Cytokine genes used are H-IL-2, G-IFN-γ, C-IL-2+GM-CSF and F-untreated.

## EXAMPLE 1

Materials and methods

PCMVlacZ was obtained from Clontech, Palo Alto, CA, USA. The murine transitional cell carcinoma cell line MB49 is available from the University of Iowa.

## EXAMPLE 2

*In vitro assay for DMBC transfection efficiency*

$2x10^5$ cells were plated on coverslips in 6 well tissue culture plates in RPMI 1640 supplemented with 10% FBS, 200mM L-glutamine (Sigma), 5000U/ml penicillin and 5mg/ml streptomycin (Sigma). Cells were washed twice with 1XPBS and transfected with 7.5ug of pCMVlacZ complexed with 20ug of DOTAP (Boehringer Mannheim, Germany) and 40ug of cholesterol (Sigma). The complexes were formed by making up the DNA and DMBC to 165ul with 20mM Hepes buffer (Gibco BRL) and mixing for 15 minutes. This was made up to 1ml with RPMI 1640, and added to wells for the appropriate length of time, washed and replaced with 3ml of fresh RPMI 1640. After approximately 48h, the cells were washed with PBS and fixed in 0.05% glutaraldehyde (GTA), 1mM magnesium chloride in phosphate-buffered saline (PBS), then stained with X-gal (1mg/ml in 5mM potassium ferricynaide and 5mM potassium ferrocynaide, 2mM magnesium chloride, in Tris buffer (pH 8.5) overnight[33]. Transfection efficiency was determined by counting the number of blue colonies relative to unstained cells in four quadrants on each slide and taking the average of these results. Transfections were performed in duplicates and repeated twice.

## EXAMPLE 3

*Proliferation assays*

Proliferation was measured by tritiated thymidine (methyl-3H-tdR) incorporation as assayed by a liquid scintillation counter. Cells for the proliferation assay were plated at 1 x$10^4$ cells/well in a 96 well flat-bottomed plate tissue culture plate (Nunc, Rosklide, Denmark) and transfected at optimal conditions as described above for the appropriate length of time (2hr and 24hrs exposure), washed and replaced with fresh RPMI 1640 and incubated at 37°C. After 32 hr, supernatants were removed, and cells were rinsed with 1XPBS. Triplicate wells were incubated with 0.2µCi/ml [$^{14}$C]-thymidine (specific activity 56.5mCi/mmol; Du Pont, Wilmington, DE) for 16 hr. The plates were rinsed with 1XPBS and methanol. Then the cells were further washed twice with 5%TCA (Merck, Frankfurt, Germany) and rinsed twice with ethanol. The cells were solubilized with 1% SDS (Sigma) and radioactivity measured with a scintillation counter. [$^{14}$C]-thymidine incoporation was expressed as a percentage of the control which consisted of untransfected cells.

## EXAMPLE 4

*Detection of reporter gene expression in vivo after intravesical delivery of DNA-DMBC*

5-7 week old female C57BL6 mice were anaesthetised, their bladders were then catheterised with 24G i.v. catheter and flushed with 1X PBS. The DMBC and DNA complex were mixed for 15 minutes and diluted to a final volume of 465ul with 1X PBS and introduced intravesically. Transfection exposure times of 15 minutes, 30 minutes, 1 hour or 2 hours were evaluated. At different time points the bladders were flushed with 1X PBS to remove DNA/ DMBC complex. The mice were sacrificed 48 hours after treatment. Experiments were done in duplicates.

## EXAMPLE 5

*Histochemistry.*

Bladders, lungs, kidneys, heart, liver and spleen were removed and snap frozen in liquid nitrogen. Cryostat sections, 6 μM in thickness were fixed in 1.25% glutaraldehyde for 10 minutes at 4°C followed by incubation with X-gal for 4 hours at 37°C and were counterstained with haematoxylin[34].

## EXAMPLE 6

*DNA PCR on organs*

DNAPCR analysis employed primers unique to the pCMVlacZ. The upstream primer used was 5'-GCCGACCGCACGCCGCATCCAGC-3' and the downstream primer was 5'-CGCCGCGCCACTGGTGT-3'. PCR was carried on a total volume of 25ul containing 100ng of genomic DNA, 2ul of dNTPs (New England Biolabs), 2.5ul of reaction buffer, 1ul of each primer ( 10uM) and 1 ul of Taq polymerase (Finnzymes).The PCR program was as follows: 94°C for 30s, followed by , 60°C for 30s and 72°C for 30s for 40 cycles. PCR reactions using GADPH primers as controls for amplification. The primer sequences for gadph are upstream primer 5'-CTGCGACTTCAACAG-3' and downstream primers 5'-CACCCTGTTGCTGTAG-3'. The PCR program was as follows: 94°C for 30s, followed by , 58°C for 30s and 72°C for 30s for 40 cycles. Amplified DNA were analysed by electrophoresis on 1% agarose gel and visualised with ethidium bromide under UV light.

## EXAMPLE 7

*Transfection activity by ONPG Assay*

Cells were transfected with DMBC as above. After an incubation of 2 h the complex cells were washed twice with blank RPMI media and replaced with complete media (RPMI 1640) and the cells were further incubated for the appropriate length of time. The cells were then washed twice with 1X PBS and lysed with 150ul of lysis buffer. The protein content of the lysates was measured by the Micro BCA Protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as standard. Cell protein lysates were assayed in the reaction mixture containing and 4mg/ml of ONPG and incubated at 37°C for 1 hour when yellow color developed. Reactions were stopped by the addition of 500ul 1M $Na_2CO_3$ and $OD_{420}$ was measured.

## EXAMPLE 8

*Orthotropic implantation of tumor cells*

To establish tumors orthotropicaly, mice were anasthesized, the bladder catheterised via the urethra with a 24-gauge plastic intravenous catheter. The bladder was flushed with 1X PBS and then traumatised by instilling 0.1ml of 0.1N HCL solution and immediately neutralised by 0.1M KOH and flushed with 1XPBS. 100ul of $5x10^5$ MB49 cells were introduced intravesicaly (100ul) into the bladders of C57BL/6 female mice. Animals were examined weekly until the tumor becomes palpable, whereafter the bladders were transfected with pCMVlacZ and DMBC combination as above. Animals were sacrificed two days later, bladders removed and snap frozen in liquid nitrogen. Six-micron cryostat sections were fixed in 1.25% glutaraldehyde for 10 minutes at 4°C followed by incubation with X-gal and counterstained with haemotoxylin.

## EXAMPLE 9

*Nuclear and cytoplasmic analysis by DNA PCR*

Nuclear and cytoplasmic fractionation of transfected cells was carried out using gentle lysis with a TNE + Igepecal buffer (10mM Tris HCL, 1mM EDTA, 100mM NaCl, 1% Igepecal) followed by 7K for 5 minutes at 4°C, the cytoplasmic extract was transferred to a fresh tube. Nuclear extract was isolated using proteinase K digestion (100mM NaCl, 10Mm Tris HCL(pH8), 0.5% SDS 25mM EDTA and 0.1mg/ml proteinase K). Cells were incubated overnight at 50°C. Phenol/chloroform extraction was carried twice followed by ethanol percipitation. For DNAPCR analysis similar primers and conditions as above were used for pCMVlacZ and GADPH were used. Amplified DNA were analysed by electrophoresis on 1% agarose gel and visualised with ethidium bromide under UV light.

## EXAMPLE 10

**Results**

*In vitro transfection efficiency of non-viral agents*

The pCMVlacZ expression plasmid was used to assess the transient transfection efficiency of various non-viral agents in a 2 hour time period on MB49 cells. Both Dotap and Superfect were able to transfect MB49 cells within a 2 hour time period with efficiencies of approximately 20.4% and 14.8% respectively (Table 1). Optimal transfection was obtained using 7.5µg of DNA and 20µg Dotap. With Superfect, a much lower amount of DNA was needed to obtain similar transfection rates as Dotap namely 2 µg DNA with 7.5µl of Superfect. A very low transfection rate of approximately, 1.02% was obtained with Fugene. Neither calcium chloride (0-40µg) nor DEAE-Dextran (0-2µg) nor naked DNA could transfect MB49 cells.

To improve transfection rates the DMBC was incubated with DNA for 15 minutes prior to exposure to the cells. Addition of methylated-β-cyclodextrin containing cholesterol resulted in a 3.9 fold increase in transfection when used in combination with Dotap, and a 2.4 fold increase with Superfect (Fig. 1A and B). However, the amount of methylated-β-cyclodextrin containing cholesterol needed to produce this increase in transfection efficiency varied with the agent used. Optimal Dotap transfection was obtained with 40µg of methylated-β-cyclodextrin containing cholesterol while 10µg was optimal for Superfect. As the best transfection rates were obtained with DMBC, this combination was used in all further experiments.

**EXAMPLE 11**

*Characterisation of gene expression*

Characterisation of the β-gal gene expression as a function of time following transfection with DMBC was carried out. As shown in Figure 2a, protein expression as determined by the ONPG assay, is found within 1 h after removal of the agent and increases up to 48 hours post transfection. After 48 hours there is a steady decrease in protein expression and by 12 days post-transfection the β-gal activity had decreased by 7 fold compared to the expression at day 2 (Figure 2b). It appears that as the cells replicate the gene is lost and/or silenced.

## EXAMPLE 12

*DNA uptake following transfection*

In order to determine whether there was a difference in DNA uptake in the presence of DOTAP and DMBC, we measured the quantity of plasmid DNA extracted from cells 2h after transfection. To minimize the amount of surface bound DNA and get a better estimate of true DNA uptake, we did multiple washes with 1XPBS as well as digestion with DNAse. Figure 3, shows the PCR analysis of DNA extracted from MB49 cells. MB49 cells seem to have internalised similar amounts of plasmid DNA with both agents. Therefore, the differences observed in transfection efficiencies are not due to differential uptake of plasmid DNA but perhaps to the stability of the DNA once taken into cells.

## EXAMPLE 13

*Nuclear and cytoplasmic analysis by DNA PCR*

To determine the localisation of DNA following transfection with DOTAP and DMBC, nuclear and cytoplasmic extracts were prepared from transfected cells. The presence of DNA in these extracts were determined using PCR. In cells transfected with DMBC, the Lac Z gene was located in both the nucleus and the cytoplasmic fractions. However with Dotap alone localisation was found to be in the cytoplasm only (Figure 4).

## EXAMPLE 14

*Toxicity of DMBC /DNA complexes*

A cell proliferation assay was used to gauge the toxicity of the transfecting agents. Relative toxicity was measured by comparing proliferation in transfected and untransfected cells, 48h after plating. Cells were exposed to the transfecting agents for 2 hours and 24 hours. There was no difference in the level of thymidine incorporation between transfected and non-transfected cells exposed to either Dotap or DMBC (Figure 5A). When cells were exposed to the DMBC/DNA for 24 hours, there was a reduction in cell proliferative capacity (Figure 5B). This data seems to suggest that the combination would probably work best in organs or sites where it can be removed after a short exposure, such as the bladder.

## EXAMPLE 15

*Intravesical transfection of bladders and tumors*

In mice (n=16) which received the transfecting agents DMBC/DNA via an intravesical instillation, bladder epithelial cells facing the lumen were successfully transfected (Figure 6a and 6b) in all 16 mice. *In vivo* Dotap alone did not result in efficient transfection of bladder epithelial cells (data not shown). As well as the bladder, the lungs, kidneys, spleen heart and liver were also harvested from transfected animals and controls and were all found to stain negative for β-gal showing that transfection was limited to the bladder (Table 2). This was confirmed by PCR analysis ( Figure 7) performed on two mice. In mice, spleen cells were harvested at the same time as the bladders and the levels of CD3+, CD4+, CD8+, αβ and γδ T cells in control and transfected animals determined by flowcytometry. No difference was found between the control and experimental groups (data not shown).

Tumours were implanted in mice bladders and once established these mice were also treated intravesically with pCMVlacZ and DMBC. The superficial luminal cell layers of the hyperplasia showed β-gal staining (Figure 8). The effect of DMBC and DOTAP on transfected bladders were analysed by transmission electron microscopy. The analyses indicated that the 2h transfection exposure time with either agent did not result in any discernible structural difference in the cells facing the lumen when compared with an untransfected control bladder. There was also no indication of the accumulation of cationic lipids or cholesterol in vacuoles. Bladder epithelial cells generally contain many vacuoles but there did not appear to be an increase in the number of such vacuoles after treatment.

**EXAMPLE 16**

*Time course: in vitro and in vivo transfections*

In order to determine the transfection efficiency with shorter exposure times, the transfection complex (pCMVlacZ : DMBC) was applied to the cells for different time periods, following which it was removed and the cells cultured in complete medium for 48 h before harvesting and enzymatic analyses. The results (Figure 9a) show that β-gal activity is detectable even when the transfection time is as short as 15 min and this activity increases with the duration of exposure to the transfecting agent. There is a 4.8 fold difference in transfection rates between a 15 min exposure and a 2 hour exposure. *In vivo*, with longer exposures to the transfecting agents more of the epithelial cells facing the lumen were transfected (Figure 9b).

## EXAMPLE 17

*Duration of gene expression in vitro and in vivo*

The peak of gene expression following transient transfection was 48 hours post-transfection and reduced thereafter *in vitro*. In mice we monitored β-gal expression at 14, 21 and 30 days post transfection. By 30 days, expression was greatly reduced but still clearly detectable (Figure 10a and b). The durability of the signal could be related to the lower replication of bladder epithelial cells *in vivo* as well as presence of the construct for a longer time, ie, via nuclear localisation of the transfected gene.

## EXAMPLE 18

Our aim in the present study was to develop a non-viral system for the transfection of urothelial cells *in vitro* and *in vivo*, that would mimic the timing of conventional therapies (i.e. 2 hours) and yet ensure high transfection efficiency. We found that by introducing methylated-β-cyclodextrin containing cholesterol to our Dotap formulation we were able to achieve this. Methyl-β cyclodextrin by itself did not improve the transfection of naked DNA but in conjunction with Dotap it enhances transfection. *In vitro*, this addition gave rise to a 3.8-fold increase compared to Dotap alone. *In vivo*, we successfully transfected bladder epithelial cells facing the lumen, something we couldn't achieve with Dotap alone. Optimum transfection was obtained with a Dotap: DNA ratio of 2.7:1 (w: w) and a Dotap to cholesterol ratio of 6:1 (w: w). In this study, the cholesterol and Dotap were mixed during synthesis and this is one reason for the different amounts of cholesterol needed. When a ratio of 4:1 of Dotap to cholesterol was used the transfection efficiency decreased. The rate of diffusion of cholesterol from the methyl-

β cyclodextrin complex to the Dotap mixture will effect the amount of cholesterol that is taken up by Dotap. Furthermore, it is possible that the methyl-β cyclodextrin cholesterol complex also directly interacts with the cell membrane and thus enhances transfection.

In our study we found that although the inclusion of cholesterol enhanced transfection efficiency it had little effect on the uptake ability. However, we found that only with the improved formulation did DNA migrate to the nucleus, suggesting that the inclusion of cholesterol may play a role in the escape of DNA from endosomes thereby avoiding degradation by lysosymes.

On of the major limitations with gene therapy is the ability to deliver therapeutic genes to target cells. In this respect, the architecture of the bladder offered several advantages. Intravesical instillation of the liposome/DNA complex for just two hours, was sufficient to maximise exposure of the urothelium to the liposome/DNA complex and to ensure a high transfection efficiency, a criteria that would be important in a clinical setting for the eradication of tumours. We found that expression of the β-galactosidase gene was confined to the bladder. This makes our delivery system ideal, as it eliminates the need to worry about the transfection of other tissues *in vivo*. A similar observation was made by Shaked *et al* , who used adenoviruses to transfect the bladder[37]. However, a recent study by Hsieh Jt *et al* has questioned the effectiveness of adenovirus-based gene therapy in a clinical setting owing to the a difference in viral receptor levels observed in human bladder cancer cell lines due to transcriptional regulation of the CAR gene[38]. The advantage of the non-viral system described here is that it is not receptor dependent and should thus be applicable to all bladder tumours.

In our study we found that a two hour exposure of the DMBC/DNA complex showed no toxicity, however at 24h it did affect cell proliferation. This data seems to suggest that the combination would probably work best in organs or sites where it can be applied directly e.g. intratumoral injection or else removed after a short exposure. For our purpose the two hour time frame was sufficient to achieve high transfection efficiency in the bladder. However, the antiproliferative effect may be advantageous, as it would serve to enhance the therapeutic gene mediated killing of tumor cells. A recent study by Pinguet *et al* showed that methylated-β-cyclodextrin by itself had anti-tumor effect on human tumor xenografted in athymic nude mice[39].

Cationic liposomes have a number of important advantages over viral gene delivery systems in the clinical setting. These include the ability to use a range of gene constructs from simple plasmids to chromosomal fragments, fewer safety concerns and the ease of large scale preparations. Their principal disadvantage was their relatively lower transfection efficiency compared to viral techniques. However, with new and improved formulations[27-29], such as reported here, non-viral transfection systems may become therapeutically important.

## EXAMPLE 19

**Characterization of the effects of Dotap+methylated-β-cyclodetxrin solubilised cholesterol (DMBC) and transfected cytokine genes on MB49 cells and tumor growth**

*Surface protein expression*

Figure 11 shows a comparison of the effect of the liposomal agent alone and liposomal agent with cytokine genes namely IL2, GMCSF, IFN-γ and the combination of IL2 and GMCSF on the expression of surface proteins such as MHC class I, class II and Fas. These proteins are important mediators of immune recognition and killing of tumor cells. For the combination cytokine treatment, only 3.75 μg each of IL2 and GMCSF was used. Only IFN-γ transfection resulted in a 15.5% increase in the MHC class II expressing cells. These data indicate that 1) the cytokine genes used are functional and 2) that by using a mixture of cytokine genes the expression of surface marker proteins can be manipulated. Using half as much as IL-2 and GM-CSF resulted in increased MHC class I expression, similar to that obtained with IL-2 or GM-CSF alone, and Fas upregulation that was comparable to that obtained with GM-CSF alone. Furthermore, the liposomal agent by itself is shown to have some beneficial effects on surface marker expression on these cells.

*Animal work*

Presently, we are using our DMBC agent for intratumoral delivery of cytokine genes for the eradication of tumours established subcutaneously.

1) In an initial study we used a once weekly injection schedule to deliver six injections of two cytokines, IL2 and TNF-α and studied their ability to block tumour growth *in vivo*. There was an initial retardation of growth with IL-2 but no mice were cured. TNF-α did not inhibit tumor growth. We have previously transfected cells with this gene and

obtained measurable quantities of this cytokine by ELISA. The reasons for the lack of inhibition, have not yet been clarified.

2) The liposome preparation by itself could also decrease the growth of tumor cells *in vivo* initially, Figure 12. However, by day 37 there was not much difference between the untreated group and the liposome treated animals. The reason for this also needs to be clarified.

3) We tried a twice-weekly injection schedule for the delivery of a total of six injections in the next set of experiments (Figure 13). In this experiment we found that IFN-γ, IL2 and IL2+GMCSF had similar inhibitory effects of tumor growth. IFN-γ treatment gave a three-fold increase in the number of mice that were cured, compared to untreated mice. Surprisingly, one mouse in the untreated group did not have a visible tumor. This mouse died during the course of the experiment and displayed weight loss prior to death. It is possible that this mouse might have had a tumor that had metastasized. After 37 days we found that there was a three-fold difference in tumor size in the groups given IFN-γ, IL-2, IL-2+GMCSF when compared to the untreated group (Figure 14).

The requirement for repeated injections could be due to the fact that transfected tumor cells are destroyed but those that are not transfected keep on growing. In big tumors the liposomal reagent may not reach all the cells. Further, most mice developed ulcers at the tumor site which made it hard to deliver the liposomal agent. This indicates the important of transfecting tumor cells when the tumor is small.

Our preliminary studies in mice indicated that the transfection of tumor cells with cytokine genes can result in the abrogation of tumor growth. From our experiments listed above it would appear that the combination of IL-2+GMCSF+IFN-γ is an appropriate choice.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds

referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

## TABLE 1

### *In vitro* transfection efficiency of non-viral agents using MB49 cells

Conditions were optimised for each agent to obtain maximum transfection at 2 hr.

Table 1. In-vitro transfection

| Non-viral Agent | Type | Transfection efficiency at optimal conditions |
|---|---|---|
| Dosap | Cationic lipid | 20.4% |
| Superfect | Dendrimer | 14.8% |
| Fugene | Non-liposomal | 1.02% |
| Calcium Chloride | Chemical | 0% |
| DEAE-dextran | Chemical | 0% |

## TABLE 2

### Staining with pCMVLacZ

DMBC was limited to bladder only.

Table 2. Staining limited to bladder

| Organs | B-gal activity |
|---|---|
| Bladder | Positive |
| Lungs | Negative |
| Liver | Negative |
| Heart | Negative |
| Kidney | Negative |
| Spleen | Negative |

## TABLE 3

### Number of mice cured after each treatment

| Treatment | 1st | 2nd | 3rd | 4th | 5th | % cured |
|---|---|---|---|---|---|---|
| IFN-γ | 0 | 0 | 2/5 | 2/5 | 3/5 | 60 |
| IL-2 | 0 | 0 | 0 | 0 | 1/5 | 20 |
| GMCSF | 0 | 0 | 0 | 1/5 | 1/5 | 20 |
| IL2+GMCSF | 0 | 0 | 1/5 | 1/5 | 2/5 | 40 |
| Liposomal preparation | 0 | 0 | 1/5 | 1/5 | 1/5 | 20 |
| Untreated | 0 | 0 | 1/5 | 1/5 | 1/5 | 20 |

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DATED this 1st day of September, 1999

**National University of Singapore**

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicants

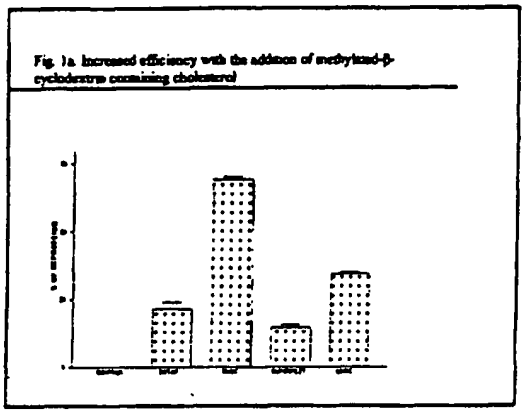
Fig. 1a Increased efficiency with the addition of methylated-β-cyclodextrin containing cholesterol

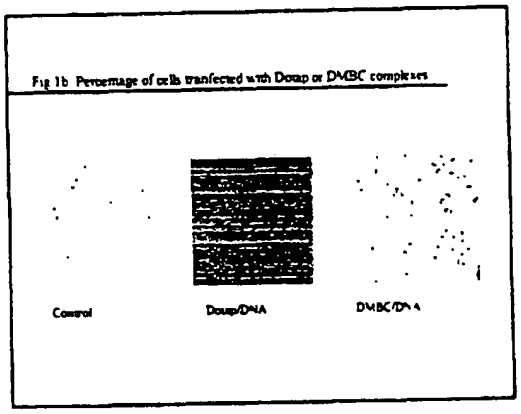
Fig 1b Percentage of cells transfected with Doxap or DMBC complexes
Control
Doxap/DNA

**FIGURE 1**

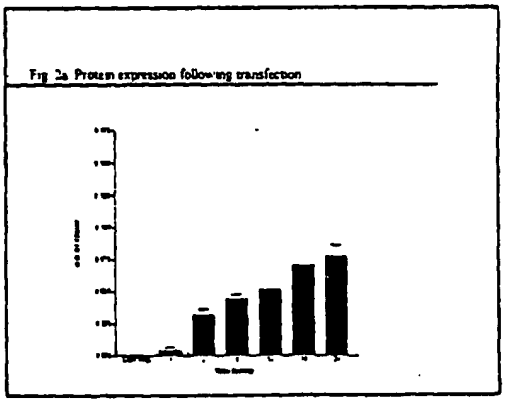
Fig 2a Protein expression following transfection

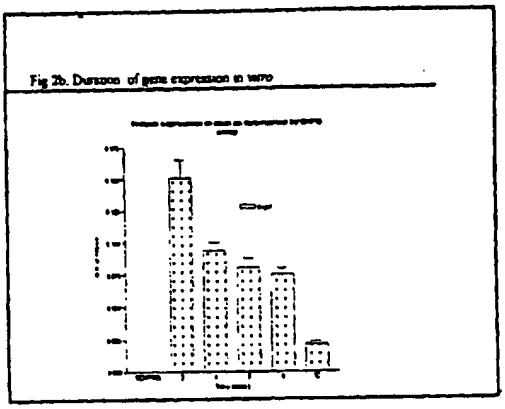
Fig 2b. Duration of gene expression in vitro

**FIGURE 2**

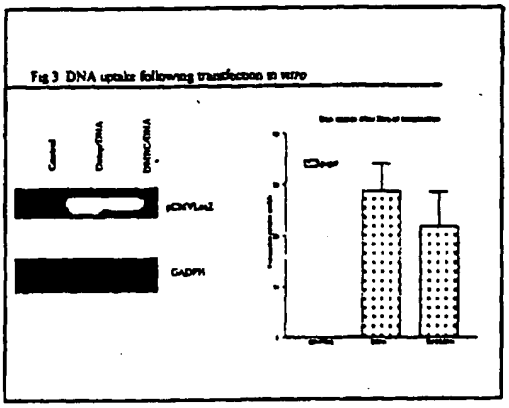


**FIGURE 3**

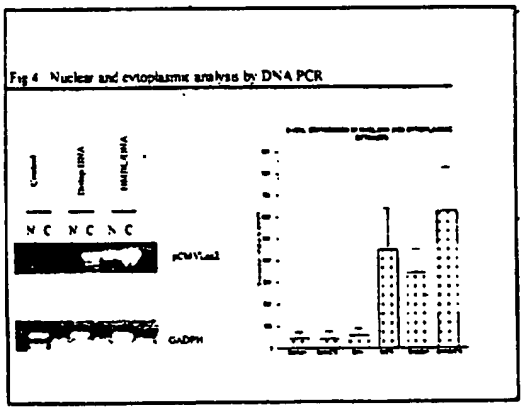
Fig 4 Nuclear and cytoplasmic analysis by DNA PCR
N C N C N C
GADPH

**FIGURE 4**

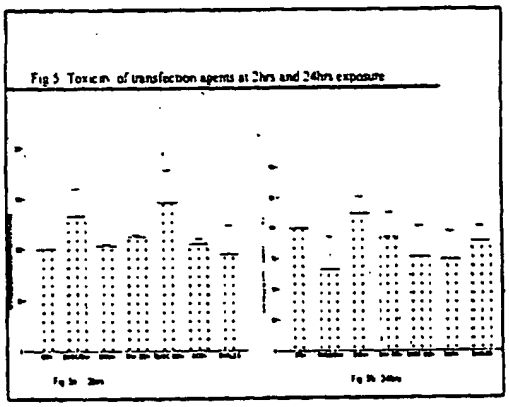
Fig 5 Toxicity of transfection agents at 2hrs and 24hrs exposure

**FIGURE 5**

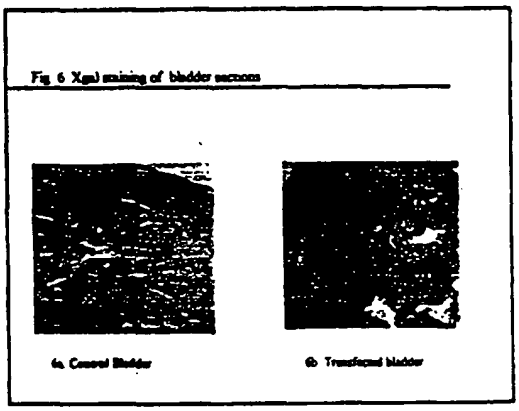
Fig. 6 X-gal staining of bladder sections
4a. Control Bladder
4b. Transfected bladder

**FIGURE 6**

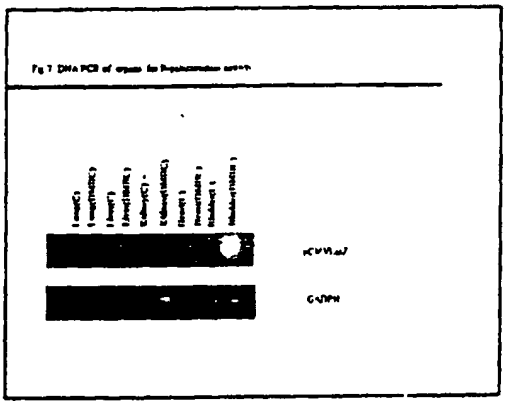

**FIGURE 7**

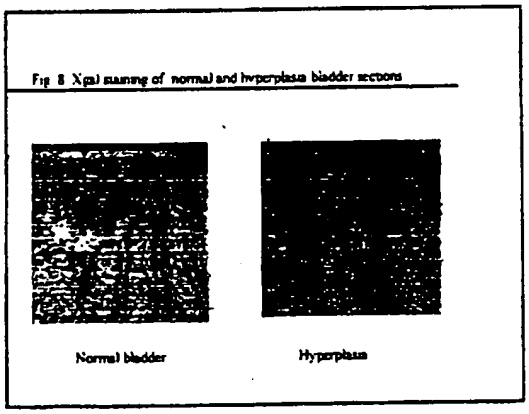
Fig 8 X-gal staining of normal and hyperplasia bladder sections
Normal bladder
Hyperplasia

**FIGURE 8**

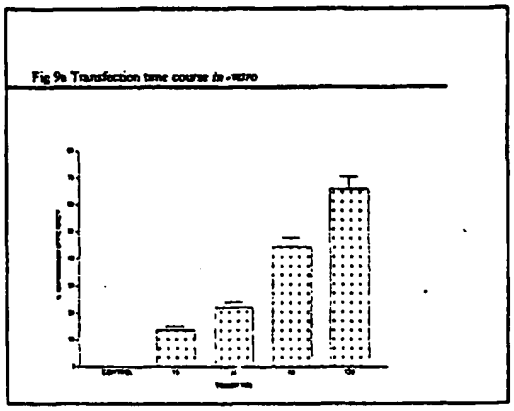
Fig 9a Transfection time course in vitro

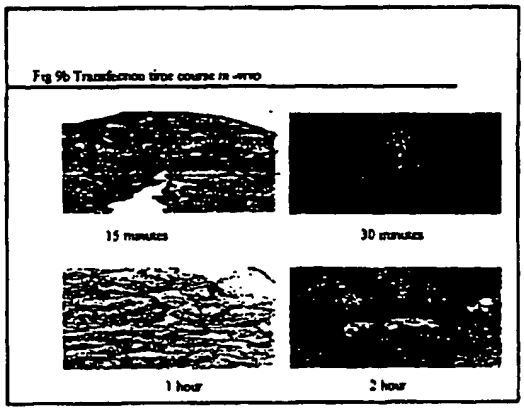
Fig 9b Transfection time course in vitro
15 minutes
30 minutes
1 hour
2 hour

**FIGURE 9**

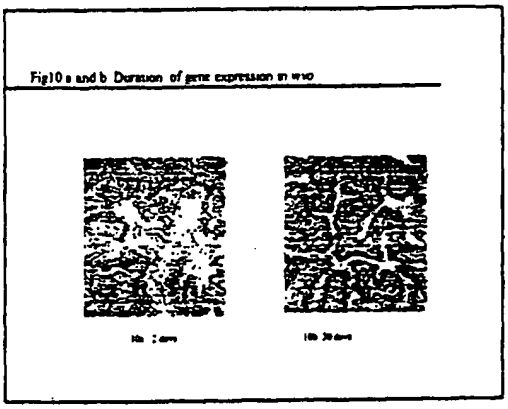

FIGURE 10

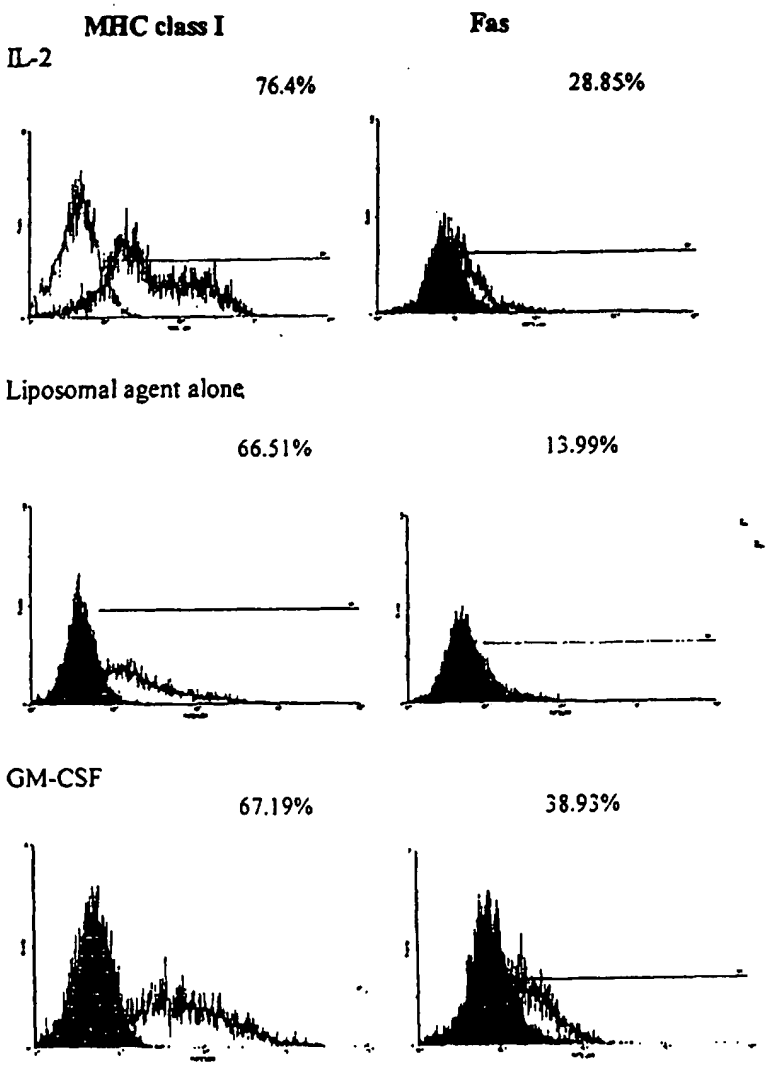
MHC class I
Fas
IL-2
76.4%
28.85%
Liposomal agent alone
66.51%
13.99%
GM-CSF
67.19%
38.93%

FIGURE 11

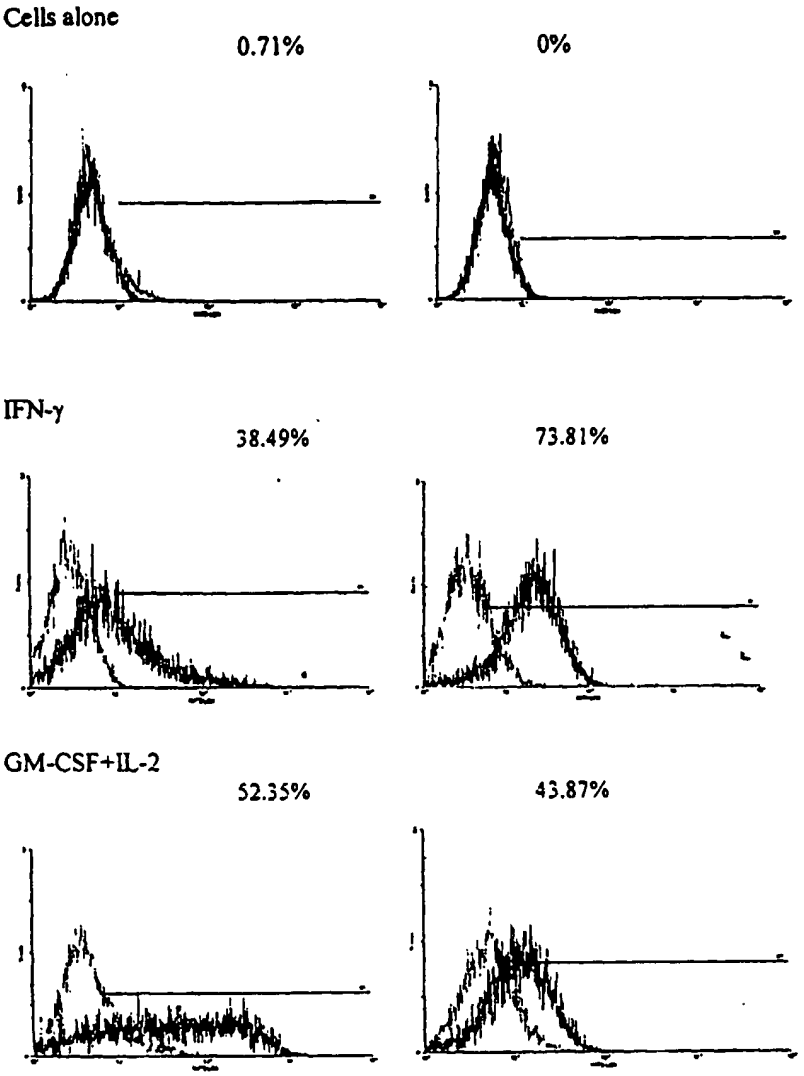


**FIGURE 11 (Continued)**

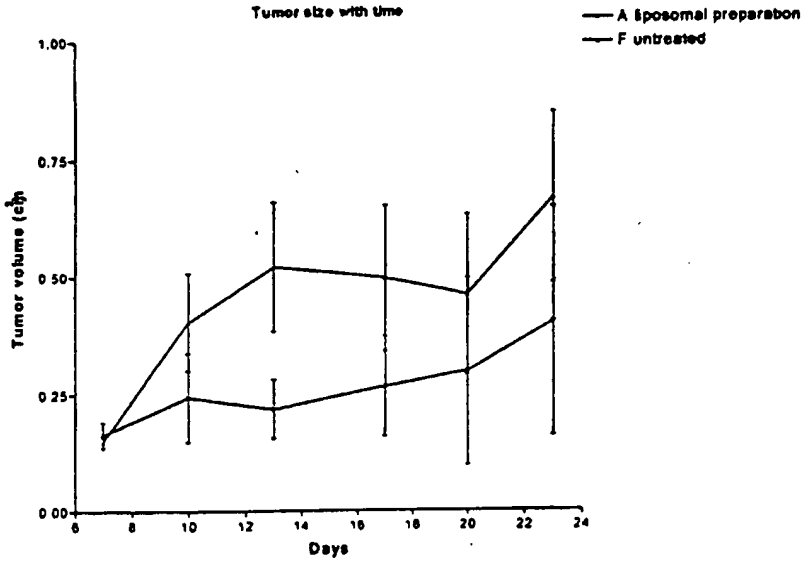
Tumor size with time
A liposomal preparation
F untreated
1.00
0.75
0.50
0.25
0.00
Tumor volume (cm³)
6
8
10
12
14
16
18
20
22
24
Days

FIGURE 12

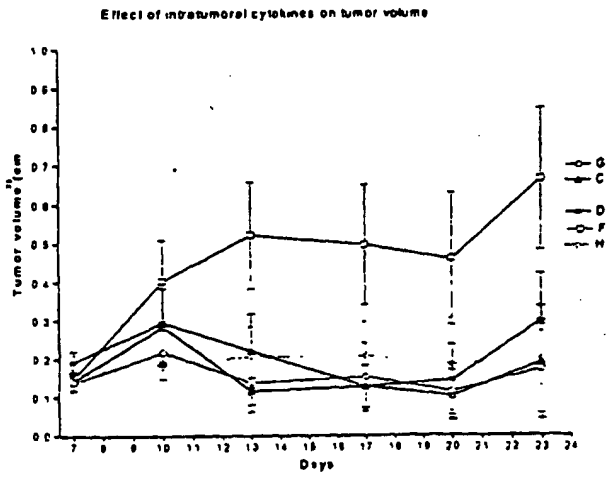
Effect of intratumoral cytokines on tumor volume
Tumor volume (cm³)
Days
G
C
D
F
H

**FIGURE 13**

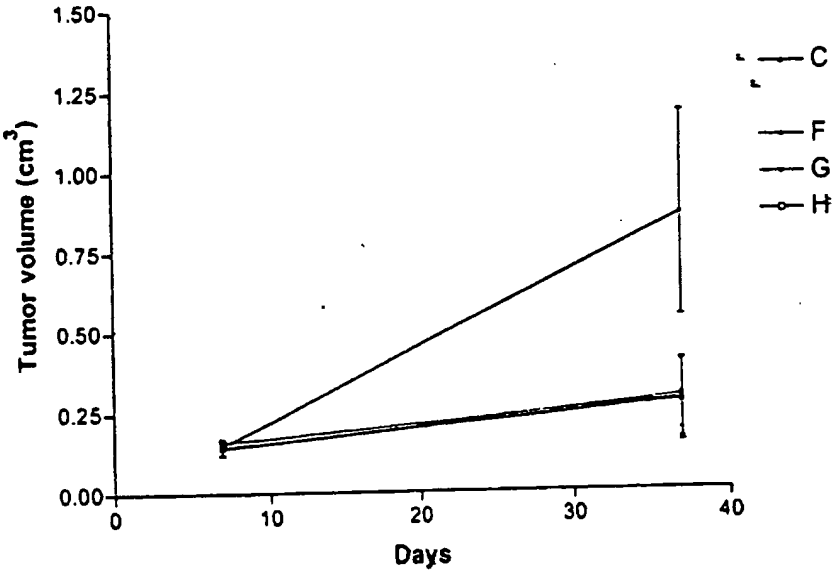
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Tumor volume (cm3)
0
10
20
30
40
Days
C
F
G
H

FIGURE 14